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Effect of Acetic Acid Vapour on the Natural Microflora and *Botrytis cinerea* of Strawberries

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Summary

To reduce losses due to microbial spoilage and to meet new food safety regulations, the optimization of postharvest treatments and storage requirements is essential in the food supply chain management. In recent years, a couple of reports have shown that treatment of several fruits with acetic acid (AA) vapour could effectively suppress fungal decay. Strawberries are one of the most perishable fruits in the market and extremely susceptible to fungal spoilage. Gray mould, caused by *Botrytis cinerea*, is the main reason for decay. Applications of AA vapour at a concentration of 2 mg l^{-1} for 30 min once, twice or three times were evaluated as fumigation treatments for strawberry disinfection. AA vapour was also applied at 4 and 6 mg l^{-1} for 30 min each. Triple fumigation with 2 mg l^{-1} AA vapour was found to be most effective treatment resulting in a 56 % reduction of decay. Alternatively, a single treatment with 6 mg l^{-1} AA vapour resulted in 12 % lower infection.

The aerobic mesophilic bacteria plate count (initial counts: $1.9 \cdot 10^4$ cfu g^{-1}) was only slightly affected by fumigation. Mould counts (initial counts: $2.0 \cdot 10^5$ cfu g^{-1}) were reduced to less than 10^3 cfu g^{-1}at an AA concentration of 3 mg l^{-1}.

Key words. emerging technology – post harvest – spoilage

Introduction

In general, fresh fruits are susceptible to postharvest infections caused by mould and bacteria. Injuries on the fruit surface often facilitate the occurrence of the decay, which results in an extensive loss of harvested fruit. In North America, losses by postharvest spoilage were estimated to be 24 % of the entire fruit production (Wilson et al. 1994). Mitcham (1996) reported loss estimations at wholesale, retail and consumer levels in the range of 28 to 41 %. In all cases the major reason for the losses was gray mould.

Gray mould induced by *Botrytis cinerea* is the main cause of decay in harvested strawberries. It is an example of a disease originating from an infection acquired in the field at an early growth stage, partially during flowering. *B. cinerea* spores can germinate in a drop of water on the petals or other parts of the flower, and later penetrate through the senescent floral parts into the edge of the receptacle of the strawberry where they develop a dormant mycelium. During ripening and storage, when the fruit's pathogen resistance decreases, the preliminary mycelium enters an active stage, and the decay develops (Powelson 1960; Jarvis 1962). The development of *B. cinerea* in stored strawberries causes a "chain" contact-infection and jeopardizes the entire basketful of fruit (Barkai-Golan 2001). Therefore, it is important to guarantee a low level of spoilage microorganisms on the fruit to minimize the risk of infection caused by the fact that an existing infection can not be reduced. This observation is supported by Eden et al. (1996) who concluded that the infection of tomato flowers by *B. cinerea* increased as a function of inoculum concentration.

The growth of *B. cinerea* can be distinctly suppressed by storage at low temperatures (0–2 °C), but will develop rapidly upon removal of the fruit to shelf-life conditions (Mitchell et al. 1996; Barkai-Golan 2001; Nunes et al. 2005). An alternative to reduce the growth of *B. cinerea* is the application of fungicidal sprays during the flowering season, but several strains of *B. cinerea* have developed resistance to these fungicides (Eckert and Ogawa 1988; Vali and Moorman 1992). To avoid resistance, five fungicides for the control of *B. cinerea* on strawberries are accredited in Germany to date (http://www.lfl.bayern.de/ips/gartenbau/11418/index.php?context=/lfl/ips/).

In recent years, a lot of alternative decontamination strategies have been investigated. Vaughn et al. (1993) and Archbold et al. (1997) reported that several volatile compounds released by strawberries during ripening could inhibit the decay caused by *B. cinerea*. Also application of the essential oil from *Thymus vulgaris* resulted in a lower deterioration of strawberries (Reddy et al. 1998). Spraying of chitosan reduced spoilage and its effectiveness rose with increasing chitosan concentration (Reddy et al. 2000). In addition microbial antagonists were applied to strawberries for biological control of gray mould (Swadling and Jeffries 1996, 1998; Helbig and Bochow 2001).

Other researchers investigated the reduction of *B. cinerea* by physical methods such as hypobaric treatment, UV-C and heat treatment (Baka et al. 1999; Romanazzi et al. 2001; Marquenie et al. 2002; Pan et al. 2004). Further-

more, the efficacy of ozone for microbial decontamination of strawberry fruit has been evaluated. Gaseous ozone was ineffective in preventing *B. cinerea* on strawberries and negatively affected the aroma of fruit (PÉREZ et al. 1999). In contrast, NADAS et al. (2003) reported a reduced decay of naturally infected strawberries, but ozone treatment also resulted in a loss of fruit aroma. In summary, all investigated methods reduce gray mould caused by *B. cinerea* to a limited extent and/or affect the strawberry fruit negatively. Therefore, the development of effective, hazard free alternative methods is of great interest.

Acetic acid (AA) is a universal metabolic intermediate occurring in plants and animals. It is commonly used by food manufacturers as antimicrobial preservative or acidulant in a variety of food products (DAVIDSON and JUNEJA 1990). The inhibitory effect of AA on microorganisms is greater than can be expected from a pH reduction alone. Undissociated AA can penetrate the microbial cell to exert its toxic effects (BANWART 1981; SHOLBERG et al. 2000). In recent years, a couple of investigations have shown that treatment of several fresh produce with AA vapour was effective in suppressing fungal decay. SHOLBERG and GAUNCE (1995) reported reduced germination of *B. cinerea* and *Penicillium expansum* on apples after AA application. The effectiveness of AA fumigation increased with relative humidity. Apparent phytotoxic effects on fruit were not observed. AA vapour fumigation significantly reduced gray mould and blue mould on sweet cherries (CHU et al. 1999, 2001). Also *Alternaria* spp. infection on cherries can be controlled by AA fumigation (SHOLBERG and GAUNCE 1996). Moreover, AA vapour has been successfully applied to pears, peaches, nectarines, apricots, plums, grapes and also strawberries (MOYLS et al. 1996; SHOLBERG and GAUNCE 1996; SHOLBERG et al. 2000, 2004; LIU et al. 2002).

These results encouraged us to study in detail, whether AA treatment may be suitable as an effective disinfectant of strawberries. Therefore, we determined the efficiency of AA vapour in preventing gray mould of strawberries caused by *B. cinerea* without any phytotoxicity to the fruit. Furthermore, the effect of AA fumigation on the native microflora of strawberries was evaluated.

Materials and Methods

For this study, organically grown strawberries (cv. 'Pegasus') were obtained from a local farmer (Potsdam, Germany) directly after harvest. All experiments were repeated three times in July 2008.

Natural strains of *B. cinerea* were collected from infected strawberries by rinsing them with sterile water. The resulting spore suspension, which included numerous species, was plated on malt extract agar (Carl Roth GmbH & Co., Karlsruhe, Germany) containing 0.04 % chloramphenicol (Fluka, BioChemika, Buchs, Switzerland) in petri dishes at 25 °C under a light bank for 7 d to promote sporulation. Then, 10 ml of sterile water was added and conidia of *B. cinerea* were aseptically removed with a sterile glass rod. The spore suspension was drawn with a sterile pipette and applied onto fruit by spraying. The final concentration on the fruit ranged from $5 \cdot 10^4$ to $1 \cdot 10^5$ spores ml^{-1} as identified with a Thoma cell counting chamber (Thoma assistant, Carl Roth GmbH & Co., Karlsruhe, Germany).

The experiments were performed immediately after purchasing of fruit and included the following investigations:

Effect of acetic acid vapour on the natural aerobic mesophilic total bacterial and mould count of strawberries

Fruit was fumigated with AA in a closed perspex box (V = 22.4 l) at 21.5 ± 0.5 °C for 30 min. The lid was fitted with a gasket and flanged to permit hermetic sealing of the chamber (Fig. 1). The chamber was humidified by water for 30 min before fumigation resulting in a nearly water-saturated atmosphere. Therefore, paper tissue soaked with deionized water was placed in the chamber and an axial blower (XD 8025 HS, XINDA Electric CO., LTD., Hangzhou, China) was turned on.

After removing the paper tissue 18 strawberries (3 rows of 6 fruit each) were placed on a grid in the chamber and AA (100 %, Carl Roth GmbH & Co., Karlsruhe, Germany), at three different concentrations (3, 6 and 9 mg l^{-1}), was injected with a syringe onto a filter paper through a port in the lid (Fig. 2). After the injection the axial blower, installed under the filter paper, was turned on again. During treatments the temperature and the relative humidity were kept constant.

After the treatment, aerobic mesophilic total bacterial and mould counts were analyzed. Additionally, on untreated strawberries the starting level of the aerobic mesophilic total bacterial and mould counts was also recorded.

For the determination of the aerobic mesophilic total bacterial and mould counts 5 strawberries from each treatment were mashed under sterile conditions in a stomacher for 2 min. For the analysis of total bacterial count, 10 g of the resulting slurry were added to 90 ml of Ringer solution, homogenized in a stomacher for 2 min and aliquot diluted. 100 µl of it were spread plated on Plate Count Agar (PCA, plates, Merck, Darmstadt, Germany) and incubated at 25 °C for 2 d. In the case of mould counts 10 g of the slurry were added to 90 ml of Ringer solution, homogenized in a stomacher for 2 min, aliquot diluted, plated on Rose-Bengal Chloramphenicol Agar (RBC, plates, Merck, Darmstadt, Germany) and incubated at 25 °C for 7 d.

Effect of different acetic acid concentrations on the growth of B. cinerea *on strawberries*

Fruit was inoculated with *B. cinerea* ($3 \cdot 10^5$ spores ml^{-1}) by spraying and dried at room temperature for 30 min to allow the spores to establish on the strawberry surface. Then 18 strawberries (3 rows × 6 fruits) were fumigated with AA (100 %, Carl Roth GmbH & Co., Karlsruhe, Germany, c(AA) = 0, 2, 4 and 6 mg l^{-1}) as described above. Two different treatments were investigated: fumigation with AA vapour at a concentration of 2 mg l^{-1} for 30 min once, twice or three times (Fig. 3) and application of AA vapour at 4 and 6 mg l^{-1} for 30 min each (Fig. 4). Thereafter, the strawberries were stored at 4, 16 and 22 °C in nearly water vapour-saturated air for 4 d and visually inspected at the end of the storage period. Infected strawberries were separated and the mould was identified

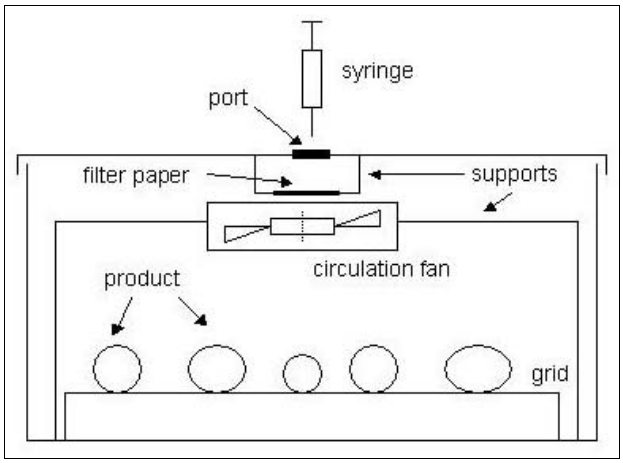


Fig. 1. Schematic representation of the fumigation chamber.

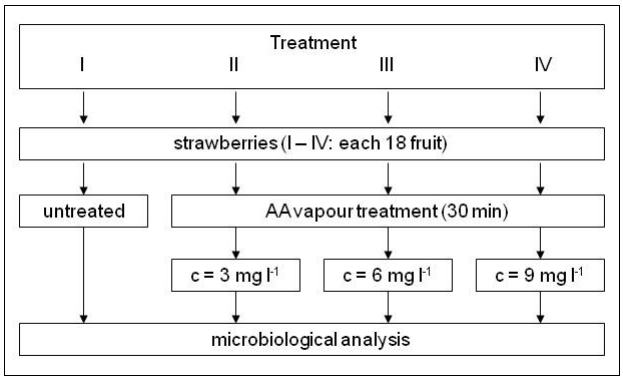


Fig. 2. Flow diagram indicating the treatment of strawberries with different AA vapour concentrations for subsequent microbiological analysis (both mesophilic bacteria and mould).

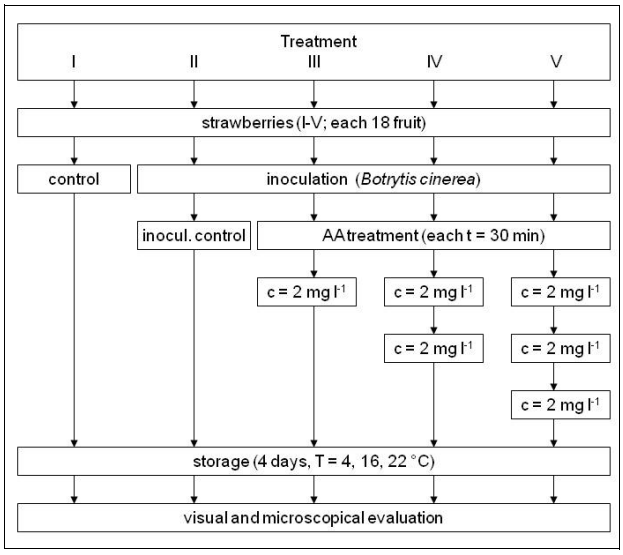


Fig. 3. Flow diagram indicating the treatment of strawberries by single, double and triple application with a constant AA vapour concentration of 2 mg l^{-1} AA vapour for subsequent visual and microscopic evaluation of gray mould control.

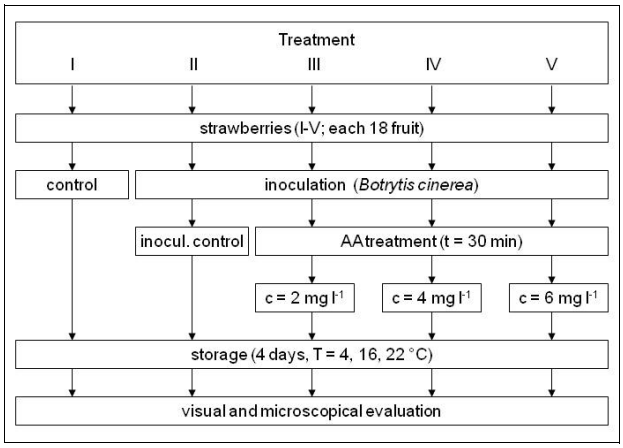


Fig. 4. Flow diagram indicating the treatment of strawberries with different AA vapour concentrations, each of the single treatments for subsequent visual and microscopic evaluation of gray mould control.

microscopically. The determinations were performed three times.

Statistical Analysis

Data were statistically analysed with WinSTAT (R. Fitch Software, Bad Krozingen, Germany). Treatments means were statistically compared using the Duncan's multiple range test ($P < 0.05$).

Results

Effect of acetic acid vapour on the native aerobic mesophilic total bacterial and mould counts of strawberries

The initial native aerobic mesophilic total bacterial count of the strawberries was $1.9 \cdot 10^4$ cfu g^{-1} fresh mass. Up to an AA vapour concentration of 6 mg l^{-1} no significant effects on the density of the microbial population and the appearance of strawberries were observed. At 9 mg l^{-1} AA aerobic mesophilic total bacterial count was reduced by half a log compared to the control, however, this reduction was statistically not significant (Fig. 5). But, the treatment resulted in necrotic spots on sepals and brown spots on the fruit body.

Applying 3 mg l^{-1} AA vapour for 30 min reduced mould counts from $2.0 \cdot 10^5$ cfu g^{-1} (starting count) to less than 10^3 cfu g^{-1}. New growth of mould was not observed during 4 d of storage at 22 °C.

Effect of different acetic acid concentrations on the growth of B. cinerea *on strawberries*

Irrespectively of the treatments, growth of *B. cinerea* on strawberries and, hence, rotting of the fruit was not

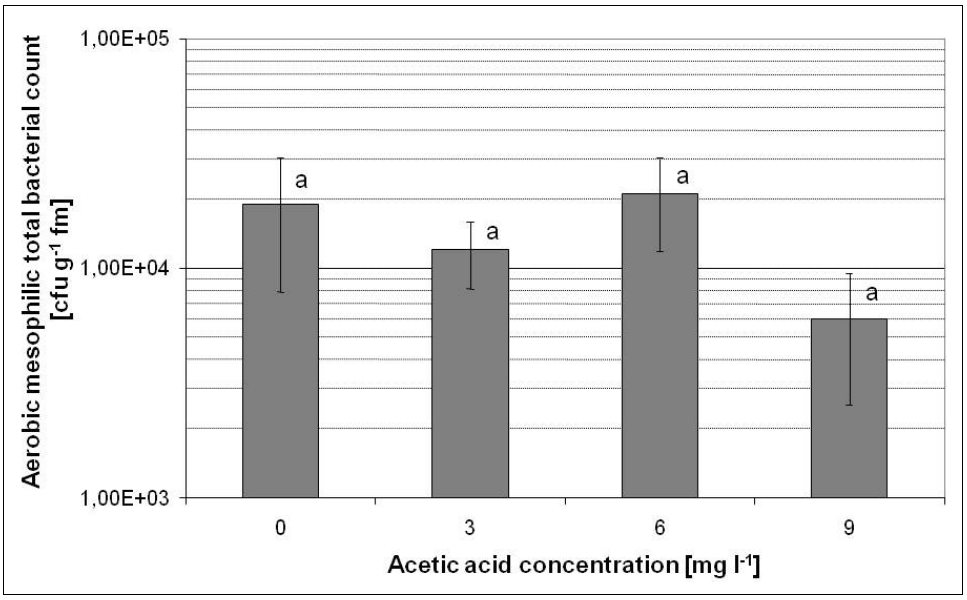


Fig. 5. Aerobic mesophilic total bacterial count of strawberries after acetic acid treatment (t = 30 min, fm = fresh mass). Bars denoted with the same letter are not significantly different from each other.

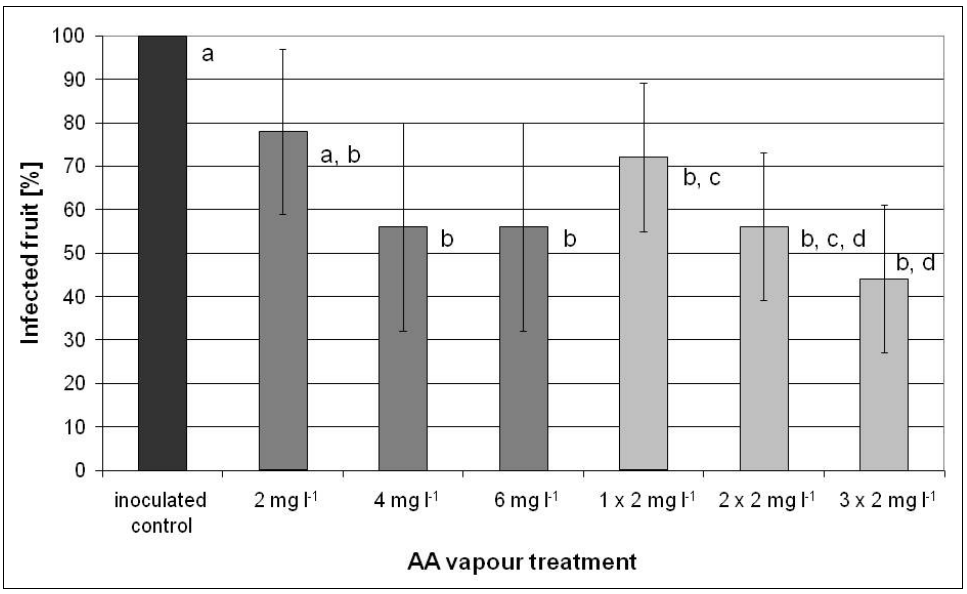


Fig. 6. Percent infection of strawberries by *B. cinerea* after single fumigation with different concentrations of AA vapour (0, 2, 4, 6 mg l^{-1}) and after repeated (single, double and triple) fumigation with 2 mg l^{-1} AA vapour after 4 d of storage at 22 °C. Bars denoted with the same letter are not significantly different from each other.

observed during 4 d at 4 and 16 °C. In contrast, at 22 °C decay of stored strawberries (recorded as percent infected fruit) was pronounced and the use of AA vapour at increasing concentrations of up to 4 mg l^{-1} resulted in an obvious reduction of decay (Fig. 6). After 4 d of storage 78 % of strawberries treated with 2 mg l^{-1} AA vapour were infected by *B. cinerea* but only 56 % of fruit were infected after initially exposing them to 4 mg l^{-1} AA vapour. Both reductions were statistically significant compared to those of the inoculated controls, infected by *B. cinerea*, which developed 100 % decay. A further increase to 6 mg l^{-1} did not further enhance control efficacy.

Repeated fumigation with 2 mg l^{-1} AA vapour resulted in a cumulative reduction effect. All reductions were significant compared to the inoculated control. The best result was observed after triple treatment. After 4 d of storage only 44 % of strawberries were infected by *B. cinerea* (Fig. 6).

The fumigation of strawberries with 2 mg l^{-1} AA was identically arranged for method I and II and resulted in comparable inhibitions of *B. cinerea* (method I: 22 % reduction, method II: 28 % reduction). An AA treatment with 4 mg l^{-1} (method I) and the double treatment of strawberries with 2 mg l^{-1} (method II) resulted in the same reduction of percent fruit infection to 56 %. The triple treatment with 2 mg l^{-1} resulted in a further inhibition of *B. cinerea* to 44 % fruit infection. However, a single treatment with 6 mg l^{-1} yielded the same percent fruit infection as the AA fumigation with 4 mg l^{-1}. Therefore, method II (treatment with 3 × 2 mg l^{-1} AA) was 12 % more effective compared to method I (treatment with 6 mg l^{-1} AA though this effect was statistically not significant.

The appearance of the infected AA-treated fruit did not differ from that of the infected controls. First, a fine mycelium was observed on the intact strawberry surface.

In a later stage of *B. cinerea* growth, the fruit became soft, and released sap.

Discussion

As a common practice, fresh strawberries are not washed before retail. Therefore methods using water with disinfectants such as chlorine or ozone can not be used for strawberry decontamination. The application of gaseous compounds such as ozone was ineffective in preventing gray mould and/or negatively affected the aroma of fruit (PÉREZ et al. 1999; NADAS et al. 2003). However, fumigation of fruit with AA vapour may be a promising alternative.

The use of AA vapour to control postharvest decay has already been discussed in literature. Canadian researchers reported that the combination of AA fumigation (5.4 mg l^{-1}) and modified-atmosphere packaging completely prevented the decay of strawberries (MOYLS et al. 1996). However, in Europe fresh strawberries are rarely sold in modified atmosphere packaging but are commonly distributed unpacked in 2.5 kg open wooden baskets, or in 0.5 kg plastic or carton punnets. In this case, the fumigation of fruit with AA vapour in normal atmosphere could provide a better control of postharvest diseases. Based on the promising results reported by MOYLS et al. (1996) the same range of AA concentrations was applied in this investigation. However, the effect of such treatment on the postharvest decay of strawberries under simulated practical conditions has not been investigated up to now.

It is known that undissociated AA can penetrate the microbial cell to exert its toxic effect, resulting in significant reduction of microbial growth and hence decay of the produce (BANWART 1981; SHOLBERG et al. 2000). Furthermore, it has been shown that AA fumigation is more effective at higher relative humidity and moderate temperature (SHOLBERG and GAUNCE 1995; DELAQUIS et al. 1999; SHOLBERG et al. 2000). Based on previous experimental data, in this study glacial AA was used to fumigate freshly harvested strawberries at room temperature in a nearly water vapour saturated atmosphere following a storage at 3 different temperature, which can be relevant during transport and sale.

The mean initial aerobic mesophilic total bacterial count of $1.9 \cdot 10^4$ cfu g^{-1} determined in our investigation on fresh untreated strawberries was in the same range as that ($7.9 \cdot 10^4$ cfu g^{-1}) reported by KOSEKI et al. (2004). No significant reduction of aerobic mesophilic total bacterial count was found after AA vapour treatment for up to 9 mg l^{-1} AA and 30 min application time. Due to the acidic nature of the produce, fungi are generally the predominant microorganisms on fruit surfaces (GOEPFERT 1980). Hence, it is not surprising that in the present study the total mould count ($2.0 \cdot 10^5$ cfu g^{-1}) was indeed significantly higher than those of aerobic mesophilic total bacteria ($1.9 \cdot 10^4$ cfu g^{-1}). As a consequence, it is very important that even moderate concentrations of AA vapour are able to reduce mould counts below their minimum detectable level. This indicates that AA vapour treatment may be successfully applied on strawberry despite the fact that AA fumigation only slightly affected native aerobic mesophilic total bacterial count. This is possible because moulds are the major causes of fruit decay.

For the experiments, which include the inoculation of strawberries, natural strains of *B. cinerea* were used. Preliminary experiments were performed with purchased laboratory strains of *B. cinerea*; these infections of the strawberries were never successful. Hence, we decided to isolate and apply naturally, strawberry derived strains, which did successfully grow on the fruit.

Furthermore, control of moulds already occurs at a relatively low AA vapour concentration (3 mg l^{-1}); and no mould counts could be detected after treatment. This is very important because an application of AA vapour at a concentration of 9 mg l^{-1} may cause necrotic spots on sepals and brown spots on the fruit body (personal observation, data not shown). SHOLBERG et al. (2004) also observed that AA fumigation may damage other fruit e.g. in pears it may induce lenticel blackening.

As indicated by the presented results low temperatures (4 and 16 °C) may considerably suppress growth of *B. cinerea* on strawberries during 4 d of storage. Similar observation was reported by other research groups (MITCHELL et al. 1996; BARKAI-GOLAN 2001; NUNES et al. 2005). Hence, in order to simulate real shelf life conditions in private households, the AA treatments of the present study were performed at 22 °C, a temperature that normally results in maximum produce losses. To increase the comparability of results, the initially applied *B. cinerea* spore concentration of 10^4 conidia ml^{-1} was in the same range as used by others, e.g. MOYLS et al. (1996).

The results demonstrate that AA vapour fumigation may successfully inhibit growth of *B. cinerea* on strawberries; however, as also clearly indicated by the presented results, a complete suppression of gray mould is not possible, at least at AA concentrations that do not harm the fruit. Furthermore, SHOLBERG et al. (2000) observed that increasing amounts of conidia on the fruit surface might reduce the effectiveness of AA treatment. Therefore, a high standard of hygiene in strawberry plantation, and during harvest and postharvest handling is an essential prerequisite for low losses in retail.

For practical applications, it must be further evaluated in detail whether short (30 min) but less (12 %) effective single treatments can economically be more meaningful than long-lasting (90 min) triple treatments. Furthermore, it is necessary to establish, whether short-term treatments may also be advantageous for the sensory quality of the treated fruit. Nevertheless, both treatments only slightly affected the natural aerobic mesophilic total bacterial flora. In addition, although AA is a stable, nontoxic, easily available and low cost compound, it is also very corrosive to metals and the equipment must consist of acid resistant material.

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